Molecular Subtyping of *Borrelia burgdorferi* in Erythema Migrans and Acrodermatitis Chronica Atrophicans

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Recently, three subtypes of *Borrelia burgdorferi* have been identified: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii,* and the VS 461 group of *Borrelia burgdorferi.* These subtypes differ by nucleotide sequence variations within several *Borrelia burgdorferi* specific genes and most likely by their pathogenetic potential. To assess whether different subtypes of *Borrelia burgdorferi* might be associated with different cutaneous manifestations and clinial courses of Lyme disease, lesional skin biopsies from 35 patients with erythema migrans and 18 patients with acrodermatitis chronica atrophicans were analyzed. A *Borrelia burgdorferi* specific gene segment encoding a 26-kD protein with subtype specific nucleotide sequence variations was amplified by a nested polymerase chain reaction technique. For molecular subtyping, the products were transcribed into complementary RNA. Upon polyacrylamide gel electrophoresis, complementary RNA molecules separate into several metastable conformational forms resulting in patterns of bands highly specific for the nucleotide sequence of the transcribed molecules. In biopsy specimens of erythema migrans, the VS 461 subtype was detected in 28 of 35 and the *Borrelia garinii* subtype in six of 35 cases. In one of 35 cases of erythema migrans *Borrelia burgdorferi* sensu stricto as well as *Borrelia garinii* was detected. In contrast, in all 18 biopsies of acrodermatitis chronica atrophicans, only the VS 461 subtype was identified. This subtype is rarely found in the USA, where acrodermatitis chronica atrophicans is almost unknown. These data indicate that acrodermatitis chronica atrophicans might be closely associated with the VS 461 group of *Borrelia burgdorferi. Key words: Borrelia burgdorferi/Lyme disease/erythema migrans/acrodermatitis chronica atrophicans/polymerase chain reaction. J Invest Dermatol 103:19–22, 1994*

Erythema migrans (EM) and acrodermatitis chronica atrophicans (ACA) represent cutaneous manifestations of an infection by *Borrelia burgdorferi (Bb),* the causative agent of Lyme disease (LD). Whereas ACA, a late manifestation of LD, is rarely found in the USA, EM, which occurs in the early course of borreliosis, is prevalent both in Europe and the USA [1,2]. Based on whole DNA hybridization techniques, recently three subtypes of *Bb* were identified: *Bb* sensu stricto, *Borrelia garinii,* and the VS 461 group of *Bb* [3,4]. These subtypes differ by the nucleotide sequence of several chromosomal (flagellin, 16S rRNA) [5–7] and plasmid genes (Osp A, Osp B) [8,9] of *Bb.* In addition, a chromosomal gene of *Bb* [10,11] encoding for a 26-kD protein [12] was identified and nucleotide sequence variations within this gene were shown to correspond to known subtypes of *Bb* [13]. The prevalence of different subtypes of *Bb* in Europe and in the USA might explain the variations in the incidence of ACA and EM in these continents [3,4,8,14,15].

Until recently, subtyping of *Bb* in clinical specimens was dependent on isolation and culture of *Bb,* which is time consuming and frequently unsuccessful. Therefore, we developed a molecular approach that allows the detection and classification of *Bb* in clinical specimens without isolating the spirochete [16]. In addition, the assay can be performed on formalin-fixed, paraffin-embedded specimens.

In this assay nucleotide sequence differences within the gene encoding for the 26-kD protein are identified by PCR amplification of a 92 base pair (bp) fragment of this gene followed by transcription of the product into complementary RNA (cRNA) and subsequent analysis by non-denaturing polyacrylamide gel electrophoresis (PAGE). Upon non-denaturing PAGE, cRNA molecules separate into several metastable conformational forms. These conformational polymorphisms strictly depend on the nucleotide sequence of the individual molecule. Analysis of single-strand conformational polymorphisms (SSCP) of cRNA molecules therefore represents a rapid and sensitive tool for identification of individual nucleotide sequence differences within PCR-amplified gene segments. This approach has been successfully applied to the detection of point mutations [17] and the subtyping of microorganisms [16] as well as to the identification of molecular marker sequences of cutaneous lymphomas [18]. In this study, molecular subtyping of *Bb* was performed in lesions of 35 patients with EM and 18 patients with ACA to determine whether different subtypes of *Bb* might be present in these skin manifestations of LD.

MATERIALS AND METHODS

Specimens Biopsy specimens of 49 patients with EM (29 fresh-frozen, cryoconserved; 20 formalin-fixed, paraffin-embedded) and of 33 patients with ACA (five fresh-frozen, cryoconserved; 28 formalin-fixed, paraffin-embedded) were obtained at the Department of Dermatology of the Ludwig-Maximilians-University of Munich. Diagnoses were based on clinical history and appearance of the lesions (EM, edematous, erythematous plaque with centrifugal growth; ACA, atrophic, thin and shrivelled skin with teleangiectasis and pigmentary changes) and on histomorphologic assessment of the biopsy specimens revealing features indicative or at least consistent with EM (normal epidermis, superficial and deep perivascular lymphocytic

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Abbreviations: ACA, acrodermatitis chronica atrophicans; *Bb, Borrelia burgdorferi*; cRNA-SSCP, complementary RNA single-strand conformational polymorphisms; EM, erythema migrans; LD, Lyme disease; Osp, outer surface protein.

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infiltrate with plasma cells) or ACA (atrophic epidermis, swelling and homogenization of collagen fibers, loss of elastic fibers). In addition, in a subset of patients, IgM and IgG serum antibodies to *Bb* were obtained to substantiate the diagnosis of a borrelial infection. Only those specimens in which *Bb*-specific DNA was amplified by polymerase chain reaction (PCR) were used for further analysis and molecular subtyping (see *Results*). As negative controls, 10 fresh-frozen and seven formalin-fixed biopsy specimens of patients with skin lesions not associated with *Bb* (lupus erythematosus, acne, granuloma annulare), as well as one formalin-fixed specimen of normal skin, were used.

Serologic Tests IgM and IgG serum antibodies to *Bb* were evaluated by an indirect immunofluorescence assay. The sera were preabsorbed for "cross-reactive" antibodies with a *Treponema phagedenis* ultrasonicate. IgM or IgG antibody titers at least 1 : 10 were regarded as being elevated [19]. The results were confirmed by a *Bb* flagellum enzyme-linked immunosorbent assay [20].

DNA Isolation Fresh-frozen biopsies were cut into small pieces with disposable razor blades and transferred into 1.65-ml microcentrifuge tubes with 100 μl of digestion buffer (0.2 M TrisCl, pH 8; 10 mM ethylenediaminetetraacetic acid (EDTA); 1% sodium dodecylsulfate) to which Proteinase K (Boehringer, Mannheim) was added to a final concentration of 1 mg/ml. After incubation at 55°C for 2 d, DNA was extracted by phenol/chloroform, ethanol preciptated and redissolved in 100 μl of destilled and autoclaved water.

DNA isolation from formalin-fixed, paraffin-embedded tissue was performed as described [21]. Briefly, ten 15-μm-thick sections were cut from each block. To avoid cross contamination, the blade of the microtome was changed between samples. The sections were deparaffinized by xylene, pelleted by centrifugation, resuspended in digestion buffer with Proteinase K, and further processed as described above for the material obtained from fresh-frozen tissue.

PCR Amplification PCR amplification of a fragment of a *Bb*-specific chromosomal gene segment encoding for a 26-kD protein was performed using a nested PCR technique as previously described [22]. Briefly, oligonucleotide primers were designed to be complementary to regions conserved between the strain B31 (*Bb* sensu stricto) and the strain G2 *(Borrelia garinii)*. First, a 171-bp fragment was amplified by an outer primer pair *Bb*-1 and *Bb*-2. Ten microliters of the DNA solution of each sample was used as template for PCR amplification reactions in a final volume of 50 μl containing 50 mM KCl, 10 mM TrisCl, 1.6 mM MgCl, 200 μM of each desoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 0.5 units of Taq DNA polymerase (Boehringer, Mannheim) and 25 pmol of each primer. The reaction mixtures were overlaid with 50 μl of mineral oil to prevent evaporation and condensation. After initial denaturation at 95°C for 5 min, 30 cycles of PCR were performed in a programmable heat block (Perkin Elmer Cetus) at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Subsequently, 0.5 μl of the product was used as template in a second PCR reaction using conditions as described above with the inner primer pair T7-*Bb*-3 and *Bb*-4. These primers amplify a 92-bp segment of the *Bb*-specific gene. The T7-RNA polymerase promoter sequence was added to the 5′ end of the T7-*Bb*-3 primer to facilitate subsequent transcription of the PCR products into cRNA. Ten microliters of the final product was analyzed by agarose gel electrophoresis (2.5% agarose in Tris-Borate–EDTA) and visualized by ultraviolet radiation after ethidium bromide staining.

Previous experiments demonstrated that PCR amplification using the primers T7-*Bb*3 and *Bb*-4 with DNA from cultured strains of spirochetes not related to LD (*Borrelia hermsii, Borrelia parkeri, Borrelia turicatae, Treponema pallidum,* and *Treponema denticola*) does not result in an appropriate PCR product. In contrast, DNA of cultured strains of *Bb* (B31, IP1, Z25, Z37, ECM1, ACA1, Bo23, Dpi, N34, 20047, Ho14, and 13 additional *Bb* strains isolated from EM lesions or ticks) was amplified and a subtype-specific pattern of bands representing conformational polymorphisms of cRNA molecules was obtained for each *Bb* subtype and confirmed by direct nucleotide sequence analysis [13]. These patterns from characterized strains of *Bb* were used as standard patterns in the present study. Primer sequences were (T7 promoter sequence underlined) for *Bb*-1, AAAACGAAGATACTCGATCTGTAATTGC; for *Bb*-2, TTGCAGAATTTGATAAAGTTGG; for T7-*Bb*-3, TAATACGACTCACTATAGGGAGATCTGTAATTGCAGAAACACCT; and for *Bb*-4, GAGTATGCTATTGATGAATTATTG.

In Vitro Transcription Three microliters of the PCR product were transcribed into cRNA by 30 units of T7-RNA-polymerase (Pharmacia) in a reaction volume of 25 μl. The transcription buffer contained 40 mM TrisCl, pH 7.5; 12 mM $MgCl_2$; 10 mM dithiothreitol; 10 mM spermidine; 1 mM of each ribonucleotide ATP, CTP, GTP, and UTP; and 7.5 units of RNA guard (Pharmacia). The mixture was incubated for 2 h at 37°C. To stop the reaction 7.5 μl of a stop solution (50% glycerol, 0.033% bromphenol blue, 50 mM NaEDTA, pH 8.0) was added.

Analysis of Conformational Polymorphisms of cRNA After heating to 65°C for 6 min, samples were chilled on ice and 15 μl of the cRNA mixture was loaded onto a 10% polyacrylamide gel with 10% glycerol. Analysis was performed in a Hoeffer 650 dual cooled page unit cooled with tap water at 16°C. The gel dimensions were 130 × 180 × 1.5 mm. Gels were run at 50 V during the first hour and at 500 V for three additional hours. After completion of electrophoresis, the wet gel was stained with ethidium bromide and photographed with Polaroid 677 film [13].

RESULTS

Detection of Borrelia burgdorferi In fresh-frozen, cryoconserved specimens *Bb*-specific DNA was detected by PCR in 26 of 29 cases of EM and in all five cases of ACA. All 10 biopsy specimens of lesions not associated with LD were negative.

In formalin-fixed, paraffin-embedded tissue samples *Bb*-specific DNA was detected in nine of 20 specimens of patients with EM and in 13 of 28 specimens of patients with ACA. All seven specimens of lesions not related to LD, as well as the formalin-fixed specimen of normal skin, were negative. In total, *Bb*-specific DNA was detected by PCR in 35 lesions of EM and 18 lesions of ACA (Table I). These samples were used for further analysis and molecular subtyping of *Bb*. The accuracy of diagnoses in these patients was substantiated by elevated IgM and/or IgG antibody titers to *Bb* in the majority of tested patients with EM and in all tested patients with ACA (Table I).

Molecular Subtyping of *Bb* The PCR products were transcribed into cRNA followed by polyacrylamide gel electrophoresis for assessment of conformational polymorphisms. Patterns obtained from the clinical samples were compared with standard patterns from the characterized cultured strains of *Bb*. In 35 lesions of EM, the VS 461 group of *Bb* was identified in 28 (80%) and *Borrelia garinii* in six (17%) cases. In one sample both *Borrelia garinii* and *Bb* sensu stricto were detected as previously reported [23]. In all 18 lesions of ACA only the VS 461 group of *Bb* was identified (Fig 1).

DISCUSSION

The system presented in this study allows the rapid molecular detection and subtyping of *Bb* in skin biopsies of patients with LD. *Bb*-specific DNA sequences were successfully detected and characterized in nearly all fresh-frozen, cryoconserved biopsy specimens of patients with EM and ACA. This high percentage of PCR-positive cases indicates that the majority of possible *Bb* subtypes associated with skin manifestations of LD will be detected by the system used. The lower percentage of PCR-positive cases in formalin-fixed, paraffin-embedded material is most likely due to technical limitations, as formalin-fixation and long-term storage in paraffin may cause extensive degradation of DNA. However, in cases where *Bb*- specific DNA sequences can be amplified by PCR from paraffin-embedded material, subtyping can be rapidly and accurately performed using the cRNA-based assay.

In comparison to the analysis of PCR-amplified gene segments by oligonucleotide hybridization or restriction enzyme digestion techniques, the system presented using conformational polymorphisms of cRNA molecules has the advantage that also novel and so far uncharacterized nucleotide sequence variations will be detected with high sensitivity. In several studies, the cRNA-based assay has proved to be able to precisely recognize even single nucleotide substitutions in analyzed gene segments [13,16,17,23]. Recently, we identified a novel and so far uncharacterized nucleotide sequence variation within the VS 461 group of *Bb* by cRNA-SSCP and subsequent confirmation by direct nucleotide sequence analysis [13,16].

Prior to the molecular analysis as presented in this study, subtyping of *Bb* in patients with LD has been performed by Western blot analyses of sera from LD patients using various borrelial strains as antigens [24]. Sera obtained from eight patients with ACA preferentially reacted with blotted proteins of the VS 461 group of *Bb*. However, serotyping of *Bb* is an indirect technique that depends on the patients immune response and that may not be equally detecta-

Table I. Characteristics of patients with erythema migrans (A) and acrodermatitis chronica atrophicans (B)[a]

	Additional Symptoms	Fixation/Storage of Biopsy[b]	Serum Antibody Titers to *Bb* (IFA-ABS)[c] IgM	IgG	*Borrelia Burgdorferi* Subtype[d]
A) Patients with erythema migrans					
1		cc	1:5	1:5	a
2	Radiculitis	cc	<1:5	1:80	b
3	Fever, multiple EM	cc	1:40	<1:5	b
4		cc	ND	ND	a
5	Arthralgia	cc	1:5	<1:5	a
6	Fever	cc	<1:5	<1:5	a
7	Myalgia	cc	<1:5	<1:5	a
8	Syncopes	cc	ND[e]	ND	a
9		cc	1:20	1:5	b
10	Myalgia, arthralgia	cc	1:40	1:20	a
11	Headache	cc	<1:5	1:20	a
12	Myalgia	cc	1:5	1:40	a
13		cc	1:5	1:40	a
14		cc	<1:5	1:20	a
15	Meningoradiculitis	cc	<1:5	1:640	b
16		cc	1:5	1:5	a
17		cc	<1:5	1:10	a
18		cc	<1:5	1:80	a
19		cc	1:20	<1:5	a
20		cc	<1:5	<1:5	a
21		cc	ND	ND	b
22	Bullous EM	cc	<1:5	1:10	a
23		cc	<1:5	<1:5	a
24		cc	1:20	1:40	a
25		ff	1:5	1:160	a
26		ff	<1:5	1:40	a
27		ff	1:20	1:20	a
28		ff	1:10	1:1280	b and c
29	Meningoradiculitis	ff	1:160	1:80	a
30		ff	<1:5	<1:5	a
31	Fatigue	ff	1:40	1:80	a
32	Fatigue	ff	1:10	1:160	a
33		ff	ND	ND	b
34	Headache	cc	<1:5	<1:5	a
35		cc	1:20	1:5	a
B) Patients with acrodermatitis chronica atrophicans					
1		cc	<1:5	1:640	a
2		cc	<1:5	1:320	a
3		cc	ND	ND	a
4	Periarticular fibrous nodules	cc	<1:5	1:1280	a
5	Arthralgia	cc	1:10	1:10	a
6	Arthralgia, myalgia, headache	ff	<1:5	1:160	a
7	Periarticular fibrous nodules	ff	1:5	1:1280	a
8		ff	<1:5	1:160	a
9		ff	<1:5	1:320	a
10		ff	<1:5	1:320	a
11		ff	1:80	1:40	a
12		ff	ND	ND	a
13		ff	ND	ND	a
14		ff	<1:5	1:80	a
15		ff	<1:5	1:640	a
16		ff	ND	ND	a
17		ff	<1:5	1:640	a
18	Arrhythmia	ff	<1:5	1:320	a

[a] In biopsy specimens of these patients *Bb*-specific DNA was detected by PCR. Molecular subtyping of *Bb* was performed by analysis of cRNA—single-strand conformational polymorphisms (cRNA-SSCP) of a PCR amplified segment of a *Bb*-specific gene with subtype specific nucleotide sequence variations.
[b] cc, fresh-frozen, cryo-conserved; ff, formalin-fixed, paraffin-embedded.
[c] Indirect immunofluorescence assay after preabsorption of the sera with Treponema phagedenis. Cutoff value for a positive test: titers at least 1:10.
[d] a, VS 461 group of *Borrelia burgdorferi;* b, *Borrelia garinii;* c, *Borrelia burgdorferi* sensu stricto.
[e] ND, not done.

ble and specific in all cases during the course of the disease. Furthermore, retrospective studies on cases with only formalin-fixed, paraffin-embedded biopsy material available are not possible. In another study using a classification system for *Bb* based on the reactivity of monoclonal antibodies for OspA with cultured strains of *Bb*, 10 of 11 strains originally isolated from ACA lesions were classified as the VS 461 group of *Bb* [14].

The observation of an association of the VS 461 subtype of *Bb* with ACA might explain the exceedingly low incidence of ACA in the USA as compared to the considerably higher number of cases of ACA in Europe [1,2]. In the USA *Bb* strains isolated from skin biopsies of patients with LD as well as other sources, e.g., ticks or cerebrospinal fluid, so far all belong to the *Bb* sensu stricto group, whereas three subtypes of *Bb* including the VS 461 group are

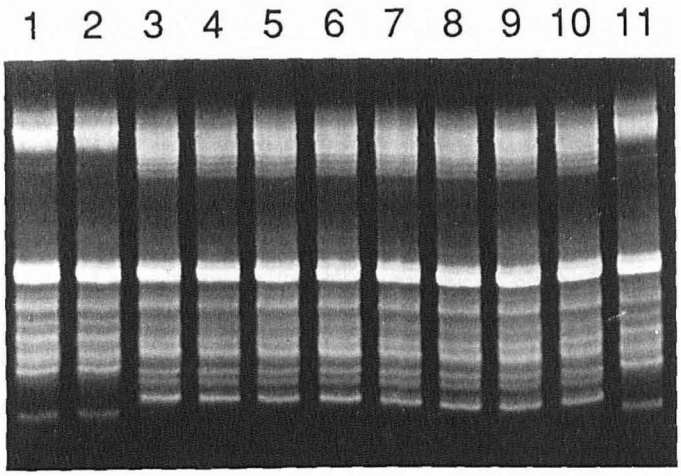

Figure 1. Representative examples of molecular subtyping of *Bb* in specimens of patients with EM *(lanes 1–5)* and ACA *(lanes 6–9)*. A segment of a *Bb*-specific gene with subtype-specific nucleotide sequence variations was amplified by PCR. Molecular subtyping was performed by analysis of cRNA–single-strand conformational polymorphisms (cRNA-SSCP) of the amplified gene segment by PAGE. *Lane 10:* standard pattern obtained from cultured strain ECM 1 (VS 461 subgroup of *Bb*). *Lane 11:* standard pattern obtained from cultured strain N34 *(Borrelia garinii)*. In biopsy specimens of patients with EM the *Borrelia garinii* subtype *(lanes 1–2)* or the VS 461 group of *Bb (lanes 3–5)* was detected. In all biopsy specimens of patients with ACA only the VS 461 group of *Bb* was detected *(lanes 6–9)*.

present in Europe [3,4,8,14,15]. Further studies using rapid and sensitive assays such as the cRNA-SSCP method presented here will help to clarify further associations of specific subtypes of *Bb* with distinct clinical courses of LD, e.g., arthritis or meningoencephalitis. They may play a future role in the rational management and follow up of patients with LD, as subgroups of patients with risks for specific long-term complications of LD such as ACA can be identified.

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